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(54) Title: SERUM AMYLOID A ISOFORM FROM COLOSTRUM

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                                                    ▼
M  N  L  S  T  G  I  I  F  C  F  L  I  L  G  V  S  S  Q  R
ATGAACCTTTCCACGGGCATCATTTTCTGCTTCCTGATCCTGGGCGTCAGCAGCCAGAGA 60
TACTTGGAAAGGTGCCCGTAGTAAAAGACGAAGGACTAGGACCCGCAGTCGTCGGTCTCT

W  G  T  F  L  K  E  A  G  Q  G  A  K  D  M  W  R  A  Y  Q
TGGGGGACATTCCTCAAGGAAGCTGGTCAAGGGGCTAAAGACATGTGGAGAGCTTACCAA 120
ACCCCCTGTAAGGAGTTCCTTCGACCAGTTCCCCGATTTCTGTACACCTCTCGAATGGTT

D  M  K  E  A  N  Y  R  G  A  D  K  Y  F  H  A  R  G  N  Y
GACATGAAAGAAGCCAACTACAGGGGTGCAGACAAATACTTCCACGCCCGTGGAAACTAT 180
CTGTACTTTCTTCGGTTGATGTCCCCACGTCTGTTTATGAAGGTGCGGGCACCTTTGATA

D  A  A  R  R  G  P  G  G  A  W  A  A  K  V  I  S  N  A  R
GACGCTGCCCGAAGGGGACCTGGGGGTGCCTGGGCTGCTAAAGTGATCAGTAACGCCAGA 240
CTGCGACGGGCTTCCCCTGGACCCCCACGGACCCGACGATTTCACTAGTCATTGCGGTCT

E  T  I  Q  G  I  T  D  P  L  F  K  G  M  T  R  D  Q  V  R
GAGACTATTCAGGGAATCACAGACCCTCTGTTTAAGGGTATGACCAGGGACCAGGTACGG 300
CTCTGATAAGTCCCTTAGTGTCTGGGAGACAAATTCCCATACTGGTCCCTGGTCCATGCC

E  D  S  K  A  D  Q  F  A  N  E  W  G  R  S  G  K  D  P  N
GAGGATTCGAAGGCCGACCAGTTTGCCAACGAATGGGGCCGGAGTGGCAAAGACCCCAAC 360
CTCCTAAGCTTCCGGCTGGTCAAACGGTTGCTTACCCCGGCCTCACCGTTTCTGGGGTTG

H  F  R  P  A  G  L  P  D  K  Y  *
CACTTCAGACCTGCTGGCCTGCCTGACAAGTACTGA 396
GTGAAGTCTGGACGACCGGACGGACTGTTCATGACT
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(57) Abstract: A novel Serum Amyloid A (SAA), isolated and purified from mammalian colostrum, is disclosed. The SAA has been isolated from colostrum of several mammalian species, including horse, cow and sheep. Nucleic acid molecules encoding the colostrum SAA, and antibodies immunologically specific for the colostrum SAA, are also disclosed.

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TITLE: SERUM AMYLOID A ISOFORM FROM COLOSTRUM

FIELD OF THE INVENTION

The present invention relates to the field of immunology and mammalian immune systems. In particular, the invention provides novel isoforms of serum amyloid A, which are found in colostrum.

BACKGROUND OF THE INVENTION

Several scientific or patent publications are referenced in this patent application to describe the state of the art to which the invention pertains. Each of these publications is incorporated by reference herein, in its entirety.

Mammals respond to tissue injury, trauma or infection by executing a complex series of biological reactions in an effort to prevent further tissue damage, to initiate repair of damaged tissue, and to isolate and destroy infective organisms. This process is referred to as the inflammatory response, the early and intermediate stages of which are referred to as the acute phase response.

The acute phase response involves a wide variety of mediators, including cytokines, interleukins and tumor necrosis factor. It also involves a radical alteration in the biosynthetic profile of the liver. Under normal circumstances, the liver synthesizes a range of plasma proteins at steady state concentrations. Some of these proteins, the "acute phase" proteins are induced in the inflammatory response to a level many times greater than levels found under normal conditions. Acute phase proteins are reviewed by Steel & Whitehead (Immunology Today 15: 81-87, 1994).

One of the massively induced acute phase proteins is Serum Amyloid A (SAA). SAA actually comprises a family of polymorphic proteins encoded by many genes in a number of mammalian species. SAAs are small apolipoproteins that accumulate and associate rapidly with high-density lipoprotein 3 (HDL3) during the acute phase of the inflammatory response. Most SAA isoforms are induced in response to inflammation; however, certain SAAs (e.g., human SAA4) appear to be constitutively expressed or minimally induced in the inflammatory response.

fragments can be defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments such as single chain Fv, chimeric antibodies (i.e., comprising constant and variable regions from different species), humanized antibodies (i.e., comprising a complementarity determining region (CDR) from a non-human source) and heteroconjugate antibodies (e.g., bispecific antibodies).

As used herein, "antisense orientation" includes reference to a duplex polynucleotide sequence that is operably linked to a promoter in an orientation where the antisense strand is transcribed. The antisense strand is sufficiently complementary to an endogenous transcription product such that translation of the endogenous transcription product is often inhibited.

As used herein, "colostrum associated serum amyloid A", "colostrum associated SAA" and/or "colostrum SAA" are used interchangeably and include but is not limited to the sequences disclosed herein, their conservatively modified variants, regardless of source and any other variants which retain the biological properties of the colostrum SAA and as demonstrated by the assays disclosed herein.

As used herein, "chromosomal region" includes reference to a length of a chromosome that may be measured by reference to the linear segment of DNA that it comprises. The chromosomal region can be defined by reference to two unique DNA sequences, i.e., markers.

The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. Every nucleic acid sequence herein that encodes a polypeptide also, by

reference to the genetic code, describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine; and UGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide of the present invention is implicit in each described polypeptide sequence and is within the scope of the present invention.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Thus, any number of amino acid residues selected from the group of integers consisting of from 1 to 15 can be so altered. Thus, for example, 1, 2, 3, 4, 5, 7, or 10 alterations can be made. Conservatively modified variants typically provide similar biological activity as the unmodified polypeptide sequence from which they are derived. For example, substrate specificity, enzyme activity, or ligand/receptor binding is generally at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the native protein for its native substrate. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See also, Creighton (1984) Proteins W.H. Freeman and Company.

By "encoding" or "encoded", with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated

regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code. However, variants of the universal code, such as are present in some plant, animal, and fungal mitochondria, the bacterium *Mycoplasma capricolum*, or the ciliate *Macronucleus*, may be used when the nucleic acid is expressed therein.

When the nucleic acid is prepared or altered synthetically, advantage can be taken of known codon preferences of the intended host where the nucleic acid is to be expressed. For example, although nucleic acid sequences of the present invention may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray *et al. Nucl. Acids Res.* 17:477-498 (1989)). Thus, the maize preferred codon for a particular amino acid may be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants are listed in Table 4 of Murray *et al., supra.*

As used herein "full-length sequence" in reference to a specified polynucleotide or its encoded protein means having the entire amino acid sequence of, a native (non-synthetic), endogenous, biologically active form of the specified protein. Methods to determine whether a sequence is full-length are well known in the art including such exemplary techniques as northern or western blots, primer extensions, S1 protection, and ribonuclease protection. See, e.g., *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997). Comparison to known full-length homologous (orthologous and/or paralogous) sequences can also be used to identify full-length sequences of the present invention. Additionally, consensus sequences typically present at the 5' and 3' untranslated regions of mRNA aid in the identification of a polynucleotide as full-length. For example, the consensus sequence ANNNNAUGG, where the underlined codon represents the N-terminal methionine, aids in determining whether the polynucleotide has a complete 5' end. Consensus sequences at the 3' end, such as polyadenylation sequences, aid in determining whether the polynucleotide has a complete 3' end.

With respect to proteins or peptides, the term "isolated protein (or peptide)" or

"isolated and purified protein (or peptide)" is sometimes used herein. This term may refer to a protein that has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form. Alternatively, this term may refer to a protein produced by expression of an isolated nucleic acid molecule.

With reference to nucleic acid molecules, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form. A heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention.

By "host cell" is meant a cell which contains a vector and supports the replication and/or expression of the vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. Preferably, host cells are monocotyledonous or dicotyledonous plant cells. A particularly preferred monocotyledonous host cell is a maize host cell.

The term "hybridization complex" includes reference to a duplex nucleic acid structure formed by two single-stranded nucleic acid sequences selectively hybridized with each other.

The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

Unless otherwise stated, the term "colostrum associated SAA encoding nucleic acid" is a nucleic acid of the present invention and means a nucleic acid comprising a polynucleotide of the present invention encoding a colostrum associated SAA. A "colostrum associated SAA gene" is a gene of the present invention and refers to a heterologous genomic form of a full-length colostrum associated SAA polynucleotide.

As used herein, "localized within the chromosomal region defined by and including" with respect to particular markers includes reference to a contiguous length of a chromosome delimited by and including the stated markers.

As used herein, "marker" includes reference to a locus on a chromosome that serves to identify a unique position on the chromosome. A "polymorphic marker" includes reference to a marker which appears in multiple forms (alleles) such that different forms of the marker, when they are present in a homologous pair, allow transmission of each of the chromosomes of that pair to be followed. A genotype may be defined by use of one or a plurality of markers.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids).

By "nucleic acid library" is meant a collection of isolated DNA or RNA molecules which comprise and substantially represent the entire transcribed fraction of a genome of a specified organism. Construction of exemplary nucleic acid libraries, such as genomic and

cDNA libraries, is taught in standard molecular biology references such as Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, Vol. 152, Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.*, *Molecular Cloning – A Laboratory Manual*, 2nd ed., Vol. 1-3 (1989); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994).

As used herein, "polynucleotide" includes reference to a deoxyribopolynucleotide, ribopolynucleotide, or analogs thereof that have the essential nature of a natural ribonucleotide in that they hybridize, under stringent hybridization conditions, to substantially the same nucleotide sequence as naturally occurring nucleotides and/or allow translation into the same amino acid(s) as the naturally occurring nucleotide(s). A polynucleotide can be full-length or a subsequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence thereof. Thus, DNAs or RNAs with backbones modified for stability or for other reasons as "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including among other things, simple and complex cells.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein, that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids. The terms "polypeptide", "peptide" and "protein" are also inclusive of modifications including, but not

198:1056) and the tryptophan (trp) promoter system (Goeddel, et al, Nucleic Acids Res (1980) 8:4057) and the lambda derived P_L promoter and N-gene ribosome binding site (Shimatake, et al, Nature (1981) 292:128).

In addition to bacteria, eucaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of *Saccharomyces cerevisiae*, Baker's yeast, are most used although a number of other strains or species are commonly available. Vectors employing, for example, the 2μ origin of replication of Broach, J.R., Meth Enz (1983) 101:307, or other yeast compatible origins of replication (see, for example, Stinchcomb, et al, Nature (1979) 282:39, Tschumper, G., et al, Gene (1980) 10:157 and Clarke, L, et al, Meth Enx (1983) 101:300) may be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess, et al, J Adv Enzyme Reg (1968) 7:149; Holland, et al, Biochemistry (1978) 17:4900). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman, et al J Biol Chem (1980) 255:2073). Other promoters, which have the additional advantage of transcription controlled by growth conditions and/or genetic background are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, the alpha factor system and enzymes responsible for maltose and galactose utilization. It is also believed terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes.

It is also, of course, possible to express genes encoding polypeptides in eucaryotic host cell cultures derived from multicellular organisms. See, for example, Axel, et al, U.S. Pat. No. 4,399,216. These systems have the additional advantage of the ability to splice out introns and thus can be used directly to express genomic fragments. Useful host cell lines include VERO and HeLa cells, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers, et al, Nature (1978) 273:113), or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses. The controllable promoter, hMT1I (Karin, M., et al, Nature (1982) 299:797-802) may also be used. General aspects of mammalian cell host system

transformations have been described by Axel (supra). It now appears, also that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in non-coding DNA regions. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes.

Transformations

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N., Proc Natl Acad Sci (USA) 1972) 69:2110, or the rbCl2 method described in Maniatis, et al, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Press, p. 254 and Hanahan, D., J Mol Biol (1983) 166:557-580 may be used for prokaryotes or other cells which contain substantial cell wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546, optionally as modified by Wigler, M., et al, Cell (1979) 16:777-785 may be used. Transformations into yeast may be carried out according to the method of Beggs, J.D. Nature (1978) 275:104-109 or of Hinnen, A., et al, Proc Natl Acad Sci (USA) (1978) 75:1929.

Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and relegated in the form desired.

The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. Typical sequences have been set forth above. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence *in vitro* starting from the individual nucleoside derivatives. The entire sequence for genes or cDNA's of sizable length, e.g., 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single

stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, M.D., Nature (1981) 292:756; Nambair, K.P., et al, Science (1984) 223:1299; Jay, Ernest, J Biol Chem (1984) 259:6311.

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by Edge, et al, Nature (supra) and Duckworth, et al, Nucleic Acids Res (1981) 9:1691 or the phosphoramidite method as described by Beaucage, S.L., and Caruthers, M.H., Tet Letts (1981) 22:1859 and Matteucci, M.D., and Caruthers, M.H., J Am Chem Soc (1981) 103:3185 and can be prepared using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7y pmoles γ32P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 μg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 μl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37° C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large

fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20° to 25° C in 50 mM Tris pH 7.6, 50 mM NaCl, 6mM MgCl2, 6 mM DTT and 0.1-1.0 mM dNTPs. The Klenow fragment fills in at 5' single-stranded overhangs but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the overhang. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or BAL-31 results in hydrolysis of any single-stranded portion.

Ligations are performed in 15-50 µl volumes under the following standard conditions and temperatures: for example, 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 µg/ml BSA, 10 mM-50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0 C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14° C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 µg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations are performed at 1 µM total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent self-ligation of the vector. Digestions are conducted at pH 8 in approximately 10 mM Tris-HCl, 1 mM EDTA using about 1 unit of BAP or CIP per µg of vector at 60° for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, re-ligation can be prevented in vectors which have been double digested by additional restriction enzyme digestion and/or separation of the unwanted fragments.

For portions of vectors derived from cDNA or genomic DNA which require sequence modifications, site specific primer directed mutagenesis may be used (Zoller, M.J., and Smith, M. Nucleic Acids Res (1982) 10:6487-6500 and Adelman, J.P., et al, DNA (1983) 2:183-193). This is conducted using a primer synthetic oligonucleotide

complementary to a single stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting partially or fully double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The resulting plaques are washed after hybridization with kinased synthetic primer at a wash temperature which permits binding of an exact match, but at which the mismatches with the original strand are sufficient to prevent binding. Plaques which hybridize with the probe are then picked, cultured, and the DNA recovered.

Verification of Construction

Correct ligations for plasmid construction can be confirmed by first transforming *E. coli* strain MC1061 obtained from Dr. M. Casadaban (Casadaban, M., et al, J Mol Biol (1980) 138:179-207) or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance by using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D.B., et al, Proc Natl Acad Sci (USA) (1969) 62:1159, optionally following chloramphenicol amplification (Clewell, D.B., J Bacteriol (1972) 110:667). Several mini DNA preps are commonly used, e.g., Holmes, D.S., et al, Anal Biochem Acids Res (1979) 7:1513-1523. The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy nucleotide method of Sanger, F., et al, Proc Natl Acad Sci (USA) (1977) 74:5463 as further described by Messing, et al, Nucleic Acids Res (1981) 9:309, o4 by the method of Maxam, et al, Methods in Enzymology (1980) 65:499.

Hosts Exemplified

Host strains used in cloning and prokaryotic expression herein are as follows:

For cloning and sequencing, and for expression of construction under control of most bacterial promoters, *E. coli* strains such as MC1061, DH1, RR1, C600hfl, K803, HB101, JA221, and JM101 can be used.

3. Assays Based on the Discovery Of SAA in Colostrum

The discovery of a specific, constitutively expressed form of SAA in colostrum enables a new way of detecting the presence of colostrum in a sample containing a mixture of biological fluids (e.g., colostrum and milk). For instance, since SAA is elevated in colostrum and not in milk from normal mammary tissue, the measurement colostrum SAA in a milk sample can be used to differentiate colostrum from milk. Accordingly, in instances where it is undesirable to have milk that contains colostrum (some countries have laws to this effect), an immunological or hybridization assay, as described above, may be used to detect colostrum-tainted milk. Accordingly, in instances where it is undesirable to have milk that contains colostrum, an immunological or hybrization assay, as described above, may be used to detect colostrum tainted milk.

Colostrum SAA also may be used for a variety of other purposes. These include, but are not limited to its use as (1) a carrier for delivery of molecules across the gut or vasculature, (2) a nutritional supplement for development of the gut mucosa in newborns, and (3) as a regulator of immune responses (via injection or oral administration).

4. Pharmaceutical Preparations

According to the invention Applicant has discovered that the colostrum associated SAA of the invention and more particularly its active site, (the TFLK motif) stimulate mucin production in the intestine. This is significant as mucins have been shown to have a key role in the prevention and treatment of intestinal infections and many probiotics act through inducing mucin production. See Mack et al, "Probiotics inhibit enteropathogenic *Escherechia coli* adherence in vitro by inducing intestinal mucin gene expression", 1999, Am J Physiol, 4 Part 1 G941-950, the disclosure of which is incorporated herein by reference. Thus the invention also includes pharmaceutical preparations for animals involving colostrum associated SAA. Those skilled in the medical arts will readily appreciate that the doses and schedules of pharmaceutical composition will vary depending on the age, health, sex, size and weight of the animal rather than administration, etc. These parameters can be determined for each system by well-established procedures and analysis e.g., in phase I, II and III clinical trials.

For administration, the colostrum associated SAA can be combined with a pharmaceutically acceptable carrier such as a suitable liquid vehicle or excipient and an optional auxiliary additive or additives. The liquid vehicles and excipients are conventional and are commercially available. Illustrative thereof are distilled water, physiological saline, aqueous solutions of dextrose and the like.

In general, in addition to the active compounds, the pharmaceutical compositions of this invention may contain suitable excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Oral dosage forms encompass tablets, dragees, and capsules. Preparations which can be administered rectally include suppositories. Other dosage forms include suitable solutions for administration parenterally or orally, and compositions which can be administered buccally or sublingually.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself well known in the art. For example the pharmaceutical preparations may be made by means of conventional mixing, granulating, dragee-making, dissolving, lyophilizing processes. The processes to be used will depend ultimately on the physical properties of the active ingredient used.

Suitable excipients are, in particular, fillers such as sugars for example, lactose or sucrose mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example, tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch, paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added, such as the above-mentioned starches as well as carboxymethyl starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are flow-regulating agents and lubricants, for example, such as silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate and/or polyethylene glycol. Dragee cores may be provided with suitable coatings which, if desired, may be resistant to gastric juices.

For this purpose concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium

dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetylcellulose phthalate or hydroxypropylmethylcellulose phthalate, dyestuffs and pigments may be added to the tablet of dragee coatings, for example, for identification or in order to characterize different combination of compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition stabilizers may be added. Possible pharmaceutical preparations which can be used rectally include, for example, suppositories, which consist of a combination of the active compounds with the suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, paraffinhydrocarbons, polyethylene glycols, or higher alkanols. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base material include for example liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of active compounds in water-soluble or water-dispersible form. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, include for example, sodium carboxymethyl cellulose, sorbitol and/or dextran, optionally the suspension may also contain stabilizers.

In addition to administration with conventional carriers, active ingredients may be administered by a variety of specialized delivery drug techniques which are known to those of skill in the art. The following examples are given for illustrative purposes only and are in no way intended to limit the invention.

As used herein the term "an effective amount" shall mean an amount of colostrum associated SAA sufficient to increase mucin production so that adherence of pathogens to mucosal cells is decreased as determined by the methods and protocols disclosed herein.

According to the invention, the novel colostrum associated SAA and more particularly its TFLK motif active site has been shown to stimulate mucin production, more specifically MUC3. Mucin production has been shown to inhibit the adherence of *E coli*, and probiotic agents which do the same, have been shown to work through stimulation of mucins. The colostrum associated SAA and/or peptide can be used as a probiotic.

The significance of mucins in intestinal infections lies in their ability to PREVENT the events necessary for infectious organisms to cause disease.

Mucins are produced by intestinal epithelial cells and secreted onto their surface. Thus, mucins are strategically located between the epithelial cells of the gut and offending agents ingested into the intestinal tract (i.e. infectious agents, noxious substances).

Mucins also inhibit the adherence of bacteria to the epithelial cells of the intestinal tract. Binding of bacteria to the lining cells of the gut is the first step in invasion, toxin delivery and development of diarrheal disease. If binding of the enteric pathogens is inhibited then disease does not develop.

Mucins have been shown to inhibit replication of viruses.

Mucins are part of innate immunity and a basic defense system of the gut. Thus, in comparison to the antibody/T-cell driven acquired immune system, mucins provide advantages including: immediate or rapidly inducible response to offending agents; broad spectrum of action; locally effective; intact across animal kingdom.

Increased production of mucins are possible by influences outside of the intestinal cell.

Increased mucin secretion due to infectious agents is a well-known clinical phenomenon. Mucin inhibits infectious intestinal bacteria from attaching to intestinal cells and thus, prevents infection. This is accomplished by mucins attaching to the structures on the wall of the bacteria that would normally be used to attach to the cells. Probiotic bacteria (non-infectious bacteria) prevent attachment of infectious intestinal bacteria to epithelial cells lining the intestinal tract. Secreted material from probiotic bacteria cause intestinal cells to produce more mucin and is the mechanism whereby probiotic agents

prevent infection.

Applicant has demonstrated that colostrum associated SAA is present in the colostrum of mammalian species and is produced by ductal epithelial cells of the mammary gland. Further, by amino acid sequence analysis, that a portion of colostrum associated SAA is preserved among many animal species. Applicant has synthesized a 10 amino acid peptide from bovine colostrum associated SAA, which contains the TFLK motif, species-preserved region of the molecule. This peptide increases the production of MUC3 in cells isolated from the small intestine by activating the gene responsible for mucin production. Applicant has shown that the intestinal mucin genes are turned on very rapidly (within 30 minutes) by this peptide. Further, the increase in mucin production by this peptide is related to its concentration around the intestinal cells. Experiments show low concentrations of colostrum associated SAA peptide will not cause an increase in mucin production whereas too much colostrum associated SAA peptide will decrease the gene driven production of mucin. This phenomenon is very common in biological systems and shows that it is a specific dose dependent effect. A four amino acid region "TFLK" and the specific order of those four amino acids within the colostrum associated SAA peptide are responsible for stimulating mucin production. It has also been shown that other peptides of the amyloid molecule unrelated to the unique colostrum associated SAA region do not stimulate mucin production.

This demonstrates pharmaceutical applications of this peptide for numerous enteric pathologies. For example the prevention of traveler's diarrhea. Many infectious organisms are geographical in nature and travelers outside of their own areas have usually not been previously exposed to these organisms, thus have not developed immunity to them. Many people will take antibiotics before traveling, but some antibiotics have deleterious side effects and also organisms are becoming resistant to many antibiotics.

Another other potential use would be to prevent dysentery and other infectious diseases particularly for the military. Vaccine development is proving to be problematic. For example, failure of military recruits to take vaccination (anthrax vaccine) and disease caused by vaccinations leading to the removal from the market of the vaccine (rotavirus vaccine). Colostrum associated SAA is to be a rapid, safe and effective means to reduce or prevent intestinal-related infections.

Another example includes prevention or treatment of infant diarrhea. Breast fed infants have far fewer infections than formula fed infants. Since colostrum is a natural substance which is beneficial to the infant and colostrum associated SAA is a component of colostrum, it will be an invaluable natural addition to formula. Such formulas are commonly commercially available such as Infamel™, Similac™, Carnation Good Start™, and Gerber™. Probiotics have been shown to reduce severity and shorten the recovery time for viral caused diarrhea. Thus, another use for colostrum associated SAA would be for children with this condition which would also have an economic impact by reducing hospital stays and costs.

Yet another example includes the prevention or treatment of necrotizing enterocolitis (NEC). This is a serious complication that occurs in premature infants. With the various reproduction techniques that are being used there has been an explosion in the number of premature infants. Therapy for NEC has remained the same for the last few decades. Since bacteria in the gut of the premature infant have a major role in the development of NEC, therapy for this condition consists of keeping the infant from feeding, giving strong antibiotics and hoping that the bowel does not perforate.

Another example includes the prevention of diarrhea in areas of outbreaks. *E. coli* 0157:H7 outbreaks causing which can lead to deaths from hemolytic-uremic syndrome. We have shown that mucin production prevents *E. coli* from adhering to epithelial cells and thus could prevent this infection.

Yet another example includes the treatment or prevention of urinary tract infections. The bladder epithelial cells are very similar to intestinal epithelial cells and are capable of producing mucins. Therefore prevention of urinary infections, including hospitalized patients with urinary catheters, would also be a use for the pharmaceutical compositions of the invention.

Yet another example includes veterinary medicine, for the prevention of infectious diarrhea in herd animals to allow for removal of antibiotics from the feed.

Although this disclosure includes upregulation of intestinal mucins, epithelial cells lining other mucosal surfaces, (e.g. nasopharynx, bladder, etc.), also produce mucins. These mucins function to prevent infections analogous to intestinal mucins, and would also be effective targets for treatment according to the invention.

The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

EXAMPLE 1

Comparative Analysis of SAA in Serum, Colostrum and Whey

The purpose of this study was to determine if SAA levels in colostrum and whey corresponded to serum SAA levels in mastitis symptomless and symptomatic cows.

Colostrum, whey and serum samples were obtained from a challenge model study in which cattle were vaccinated against a gram positive organism. Two sets of samples were utilized: one set (4 cows) from vaccinated animals that displayed clinical symptoms of mastitis, and the other set (5 cows) from vaccinated animals showing no clinical symptoms. Sample designations are shown below:

Vaccinates - Non Clinical (NC)	Vaccinates - Clinical (C)
NC Cow A	C Cow A
NC Cow B	C Cow B
NC Cow C	C Cow C
NC Cow D	C Cow D
NC Cow E	

Whey/colostrum samples were obtained from the quarter displaying the clinical symptoms.

ELISA assays were conducted according to standard protocols, e.g., as described by McDonald et al. (J. Immunol. Meth. 144: 149-155, 1991), using rat anti-SAA (human) monoclonal antibodies that cross react with bovine SAA isoforms.

Results are shown in Table 1:

Table 1:

Comparison of Bovine Mastitis Sera, Whey and Colostrum SAA values

Nonclinical Samples:			Clinical Samples:		
Sample	Sera	Whey/Colost.	Sample	Sera	Whey/Colost.
	ug/ml	ug/ml		ug/ml	ug/ml
NCA Day 0	3.9	2.3	CA Day 0	1.3	0.8
NCA Day 14	1.4		CA Day 14	1.9	
NCA Day 28	1.0		CA Day 28	1.1	
NCA Day 42	1.1		CA Day 42	0.8	
NCA calving	13.6	117.8	CA Calving	3.6	1108.0
NCA-C+14	73.0	56.5	CA C+14	12.3	0.8
NCA-C+28	11.8	3.4	CA C+28	152.6	55.0
NCB Day 0	2.6	2.3	CB Day 0	2.7	1.8
NCB Day 14	1.4		CB Day 14	21.4	
NCB Day 28	1.2		CB Day 28	3.1	
NCB Day 42	0.8		CB Day 42	1.0	
NCB calving	4.6	346.2	CB Calving	17.2	291.0
NCB-C+14	1.2	4.0	CB C+14	26.7	1.7
NCB-C+28	0.8	7.3	CB C+28	7.2	3.7
NCC Day 0	3.1	1.8	CC Day 0	1.9	1.9
NCC Day 14	1.8		CC Day 14	2.0	
NCC Day 28	1.4		CC Day 28	2.0	
NCC Day 42	0.9		CC Day 42	0.9	
NCC Calving	24.5	15.8	CC Calving	18.0	5.8
NCC C+14	6.9	1.4	CC C+14	167.9	30.0
NCC C+28	6.8	9.1	CC C+28	1.3	8.4

NCD Day 0	3.2	1.7	CD Day 0	2.7	3.4
NCD Day 14	0.9		CD Day 14	1.6	
NCD Day 28	2.4		CD Day 28	1.8	
NCD Day 42	0.8		CD Day 42	1.6	
NCD Calving	13.8	484.4	CD Calving	14.6	77.5
NCD C+14	1.3	1.6	CD C+14	1629.0	999.5
NCD C+28	1.2	5.0	CD C+28	33.1	2.5
NCE Day 0	2.6	8.0			
NCE Day 14	1.6				
NCE Day 28	1.9				
NCE Day 42	1.0				
NCE Calving	33.2	89.1			
NCE C+14	7.8	0.6			
NCE C+28	5.5	4.3			

As can be seen from the results set forth in Table 1, SAA was present in high levels in the colostrum of cows at calving in 80% of the animals tested. SAA was not detected in whey samples of most clinically healthy cows fourteen days later. SAA levels in colostrum and whey were independent of the serum concentrations of SAA. Serum levels of SAA at calving of control cows were normal (15 μg/ml), whereas the average level in the colostrum at calving was about 300 μg/ml. In one cow, the colostrum SAA was as high as 1100 μg/ml. The mastitis challenged vaccinated cow CC displayed highly elevated serum levels of SAA, but SAA levels in the whey samples were almost normal. The vaccinated challenged cow CD displayed high levels of SAA in serum and in whey.

EXAMPLE 2

Evaluation of SAA in Colostrum and Subsequent Serial Samplings of Milk

The purpose of this study was to evaluate colostrum and subsequent serial milk samplings to determine SAA content. Samples were obtained from Holstein dairy cows at the University of Nebraska - Lincoln Dairy Research Facility. Samples of colostrum were taken at calving, and subsequent milk samples were taken twice weekly for three weeks. Samples from all four udder quadrants were pooled. Results are shown in Table 2.

Table 2: SAA Levels in Colostrum and Milk Samples

Cow ID	Sample Day	SAA ug/ml
83 colostrum	Calving	184.8
83 milk	+4	0.2
83 milk	+7	0.0
83 milk	+11	0.0
83 milk	+14	0.0
83 milk	+18	0.0
83 milk	+21	0.0
908 colostrum	Calving	135.6
908 milk	+4	2.6
908 milk	+7	9.1
908 milk	+11	8.2
908 milk	+14	2.0
908 milk	+18	2.1
908 milk	+21	3.6
961 colostrum	Calving	364.6
961 milk	+4	0.3
961 milk	+7	0.5
961 milk	+11	0.0
961 milk	+14	0.0

961 milk	+18	0.0
961 milk	+21	0.0

The results show that SAA is elevated in colostrum of normal animals but in very low levels or not detected in normal milk samples after colostrum has cleared.

EXAMPLE 3

Purification of SAA from Colostrum

The procedure set forth below can be used for purification of SAA from serum, plasma, milk or colostrum from any animal species. The procedure comprises two basic steps: the SAA is purified to approximately 20% purity by hydrophobic chromatography, then further purified to approximately 95% purity by SDS-PAGE and electro-blotting.

Approximately 30 ml of octyl sepharose CL-4B (Pharmacia #17-0790-01) was prepared by washing it with approximately 10 volumes (300 ml) water to remove any traces of ethanol. This may be done by washing the gel in a sintered glass funnel (coarse, funnel volume 600 ml) or by adding the water to the gel in a beaker, and then allowing the gel to settle before pouring off the water and rewashing the gel.

The final washes (2 x 40 ml) of the gel were with a solution of 0.5M ammonium sulfate.

Prior to use, the colostrum was allowed to set at 4°C to allow the lipid layer to separate from the aqueous layer, since the lipid portion seemed to interfere with the purification procedure. After the ammonium sulfate was poured off, 20 ml of the 4°C refrigerated colostrum with elevated levels of SAA (preferably > 1µg/ml) was added to the gel (in the beaker). The suspension of colostrum and gel was swirled several times during the one hour incubation at room temperature so to allow the SAA to bind to the gel.

The gel was then poured into a 600 ml sintered glass funnel (coarse) and the non-bound fraction was collected. This non-bound fraction may be tested for SAA to determine the efficiency of binding.

The gel was washed with 5-times 50 ml 50 mM Tris, 10 mM NaCl buffer pH 7.6. The final wash should be clear.

The column was further washed with 2 X 50 ml of 30% isopropanol in Tris/NaCl.

These washes were most thorough when a syringe with a 10 gauge needle was used to eject the isopropanol/buffer solution onto the gel. The gel was thoroughly mixed when this procedure was followed.

The SAA was eluted from the gel with a solution of 60% isopropanol in TRIS/NaCl. Generally this was done in four elutions of 10 ml each.

The eluates contained a variety of proteins, of which about 20% was SAA. In samples where the SAA was too dilute it was concentrated by evaporating the isopropanol in a centrifugal concentrator. (RC 1010, Jouan Inc.)

For further purification for sequencing or amino acid analysis the proteins in the eluates were separated by SDS-PAGE and transferred to PVDF membrane by electro-blotting.

The band which was identified as SAA by SAA specific antibodies was then excised and used for sequencing.

EXAMPLE 4

ISOLATION OF COLOSTRUM ASSOCIATED SAA cDNA

RNA Isolation: Total RNA for reverse transcription-polymerase chain reaction (RT-PCR) was isolated from mammary gland epithelial cells using TRIZOL (Gibco BRL) according to manufacturers recommendations. The integrity of the RNA was checked by fractionation on a 1% (wt/vol) agarose gel and subsequent ethidium bromide staining.

First Strand cDNA Synthesis: First strand cDNA synthesis was performed using SuperScript II RNase H- Reverse Transcriptase (Gibco BRL) essentially as described by the manufacturer. Briefly, 5 μg of total RNA was mixed together with RNase-free sterile water and 20 μM of the CDNA1-T14 primer (5'-GTTGTCGACTGTAGTGGAGT$_{14}$-3') (SEQ ID NO:14) to obtain a final volume of 12 μL. The reaction mixture was incubated for 10 minutes at 75°C and then incubated at room temperature for 10 minutes. The mixture was then placed on ice while 4 μL of 5X First Strand Buffer (Gibco BRL), 2 μL of 0.1 M DTT, and 1 μL of 10 mM dNTP mix (10 mM dATP, 10 mM dGTP, 10 mM dCTP, and 10 mM dTTP at neutral pH) was added. The contents of the reaction were gently mixed and incubated at 42°C for 2 minutes. SuperScript II RNase H- Reverse Transcriptase (200 Units) was added to the reaction. Following gently mixing, the reaction

was incubated at 42°C for 1 hour. The reverse transcriptase was inactivated by heating the mixture to 70°C for 15 minutes. To remove RNA complementary to the cDNA, 2 Units of Escherichia coli RNase H (Gibco BRL) was added and the mixture was incubated at 37°C for 20 minutes. The reaction mixture was stored at -20°C until needed for second strand cDNA synthesis.

Second Strand cDNA Synthesis and Polymerase Chain Reaction: Second strand cDNA synthesis and amplification of the double stranded cDNA was performed using either Platinum Taq DNA Polymerase High Fidelity (Gibco BRL) or AmpliTaq DNA Polymerase (PE Applied Biosystems), each according the manufacturer's recommendations for the respective DNA polymerase. The PCR reactions (50 µL) with Platinum Taq DNA Polymerse High Fidelity contained 5 µg of the cDNA previously described, 20 µM of the forward primer, 20 µM of the reverse primer, 5 µL 10X High Fidelity PCR Buffer (Gibco BRL), 1 µL 10 mM dNTP mix, 2 µL 50 mM magnesium sulfate, 1 Unit Platinum Taq DNA Polymerase High Fidelity (Gibco BRL), and sterile water to obtain a final volume of 50 µL. The thermal cycling parameters with Platinum Taq DNA Polymerase High Fidelity (Gibco BRL) were 40 cycles for 30 seconds at 94°C, 15-30 seconds at 45-56oC, and 1-4 minute at 50°C.

The PCR reactions (50 µL) with AmpliTaq DNA Polymerase (PE Applied Biosystems) contained 5 µg of the cDNA previously described, 20 µM of the forward primer, 20 µM of the reverse primer, 5 µL 10X GeneAmp Buffer containing 15 mM magnesium chloride (PE Applied Biosystems), 1 µL 10 mM dNTP mix, 1.3 Units AmpliTaq DNA Polymerase (PE Applied Biosystems), and sterile water to obtain a final volume of 50 µL. The thermal cycling parameters with AmpliTaq DNA Polymerase (PE Applied Biosystems) were initiated with a hot start followed by 40 cycles forl minute at 95°C, 30 seconds at 50°C, and 1 minute at 72°C, and then 1 cycle for 15 minutes at 72°C.

The initial oligonucleotide primers suitable for PCR amplification of colostrum associated SAA cDNA were designed by back-translating the amino acid sequence obtained from colostrum associated SAA amino-terminus and tryptic digested fragments (see Fig. 2). The forward degenerate primer F1C (5'-ACNTTYCTNAARGARGCNGGNCA-3') (SEQ ID NO:15) and reverse degenerate primer R3B (5'-GAAGTGRTTGGGGTCTTTGCCACT-3') (SEQ ID NO:16), which correspond to

amino-terminal residues TFLKEAGQ (SEQ ID NO:17) and carboxy-terminal residues SGKDPNHF (SEQ ID NO:18) in the mature colostrum associated SAA protein, respectively, were used in PCR for the initial amplification of the 300 bp middle cDNA sequence for colostrum associated SAA. The 5' cDNA sequence for colostrum associated SAA was obtained by PCR and subsequent DNA sequencing using the forward primer M5RT2 (5'-AGCACAGGCAGCTCAGCTTCACCAGGA-3') (SEQ ID NO:19) and the reverse primer M5GW2 (5'-GAAGTATTTGTCTGCACCCCTGTAGTTGGCTTCTT-3') (SEQ ID NO:20). The M5RT2 primer was based on SAA cDNA sequences deposited in GenBank and the M5GW2 primer was based on the 300 bp colostrum associated SAA cDNA sequence previously described (see Fig. 2). The 3' cDNA sequence for colostrum associated SAA was obtained by PCR and subsequent DNA sequencing using the forward primer M3GW2 (5'-CTGTTTAAGGGTATGACCAGGGACCAGGTACG-3') (SEQ ID NO:21) and the reverse primer CDNA1 (5'-GTTGTCGACTGTAGTGGAG-3') (SEQ ID NO:22). The M3GW2 primer was also based on the previously described 300 bp colostrum associated SAA cDNA sequence (see Fig. 2) and the CDNA1 primer was identical to the first 19 nucleotides of the primer CDNA1-T14 used in first strand cDNA synthesis (see above for CDNA1-T14 sequence).

Cloning of colostrum associated SAA cDNA: The resulting 300 bp RT-PCR product obtained with AmpliTaq DNA Polymerase (PE Applied Biosystems) using the degenerate oligonucleotides F1C and R3B was agarose gel purified using Qiagen's QIAquick Gel Extraction Kit and cloned into Invitrogen's pCRII-TOPO vector, according to the manufacturer's recommendations. The TOPO cloning reaction was transformed into E. coli TOP10 and plated on Luria-Bertani containing 50 μg/mL kanamycin and X-Gal, as recommended by Invitrogen. Putative positive colonies were screened using the M13 Forward (-20) and M13 Reverse primer in PCR. Re-amplified inserts were fractionated in a 2% (wt/vol) agarose gel and visualized by ethidium bromide staining along with an appropriate DNA size marker.

DNA Sequencing and Computer Analysis of colostrum associated SAA cDNA: The cloned 300 bp colostrum associated SAA cDNA sequence was re-amplified with the M13 Forward (-20) and M13 Reverse primers in high-fidelity PCRs. The 5' and 3' region of the colostrum associated SAA cDNA sequence was reamplified in high-fidelity RT-

PCRs using the M5RT2/M5GW2 and M3GW2/CDNA1 primer pairs, respectively. The resulting amplicons were purified using Qiagen's Qiaquick PCR Purification System and sequenced in both directions by the DNA sequencing facility at Iowa State University (Ames, IA) in an automated ABI 377 DNA sequencer. The SP6 and T7-2 primers were used in the sequencing of the cloned 300 bp colostrum associated SAA cDNA. The primers M5RT2 and M5GW2 were used for sequencing of the 5' region of colostrum associated SAA cDNA and the primers M3GW2 and CDNA1 were used for sequencing of the 3' region of colostrum associated SAA cDNA. The DNA sequence was analyzed using the Wisconsin Genetics Computer Group (GCG) Package Version 10.1 (Madison, WI) SeqEd, PileUp, and BLASTX programs. The amino-terminal signal peptide cleavage site was identified by using the SignalP (version 1.1) program (Nielsen et al., 1997).

RT-PCR Detection of colostrum associated SAA and not Acute Phase SAA mRNA Expression by Bovine Mammary Gland Epithelial Cells: As previously described in detail, a 300 bp RT-PCR product was obtained from bovine mammary gland epithelial cells using the primer CDNA1-T14 for first strand synthesis from the mRNA present and then subsequent usage of the primers F1C and R3B for second strand synthesis and amplification. The figures show the nucleotide sequence obtained for this 300 bp RT-PCR product. This nucleotide sequence correlated with the peptide sequencing data obtained from the colostrum-associated and bovine mammary gland-associated SAA isoform (see Fig. 2).

The forward degenerate primer F2 (5'-GACATGTGGMGAGCCTACTCYGACATG-3') (SEQ ID NO:23) and reverse degenerate primer R3B (previously described) were used in RT-PCR for amplification of A-SAA cDNA. The forward primer F2 corresponds to amino-terminal residues DMWRAYSDM (SEQ ID NO:24) in the acute phase SAA (A-SAA) protein (SWISS-PROT accession number P35541) and the reverse primer R3B corresponds to the carboxy-terminal residues SGKDPNHF (SEQ ID NO:25) in both the A-SAA protein and the colostrum associated SAA protein. Subsequent cloning and nucleotide sequencing of the resulting 267 bp cDNA sequences correlated with colostrum associated SAA cDNA, strongly suggesting that colostrum associated SAA and not A-SAA transcripts were present.

The restriction endonuclease XhoI site was found to be present in the cDNA

sequence of bovine A-SAA (data not shown), but was not found in the cDNA sequence of bovine colostrum associated SAA. XhoI restriction endonuclease digestion of the 300 bp and 267 bp cDNA sequences previously described did not cleave either of these two RT-PCR products. This result additionally suggested that only colostrum associated SAA and not A-SAA mRNA was transcribed by bovine mammary gland epithelial cells.

To further verify that colostrum associated SAA and not A-SAA mRNA was expressed by the bovine mammary gland epithelial cells, the forward colostrum associated SAA -specific primer M3GW2 (previously described and shown in Fig. 2) and reverse CDNA1 primer (previously described) were again used in RT-PCR. In addition, the forward A-SAA-specific primer S3GW1 (5'-TAAGGGTACGACCAGTGGCCAGGGTCA-3') (SEQ ID NO:26), corresponding to residues FKGTTSGQGQ (SEQ ID NO:27) in mature A-SAA) and the reverse CDNA1 primer (previously described) were used in RT-PCR. However, no RT-PCR product was observed using the forward A-SAA-specific primer and reverse CDNA1 primer, further confirming no expression or the low abundance of A-SAA mRNA expression by bovine mammary gland epithelial cells.

EXAMPLE 5

COLOSTROM-SAA PRODUCTION BY BOVINE MAC-T MAMMARY EPITHELIAL CELLS

Bovine MAC-T mammary gland epithelial cells were obtained from ATCC (CRL-10274) and cultured according to recommended conditions (Turner, JD and Huynh H. Immortalized bovine mammary epithelial cell line. U.S. Pat. 5,227,301 dated July 13, 1993). MAC-T cells were cultured on Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 5 μg/ml insulin, 1μg/ml hydrocortisone and fungizone. Cells were incubated at 37°C with 5% CO_2. For colostrum-SAA production the cells were seeded onto type I Collagen coated plates. After 14 hours of incubation, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) and incubated in media (DMEM, 5 μg/ml insulin, 1 μg/ml hydrocortisone and 2.5% FCS) supplemented with prolactin from sheep pituitary gland (5 μg/ml) for the stimulation of colostrum-SAA production. Approximately one half of the media was replaced daily with

fresh prolactin supplemented media. Standard ELISA for the quantitation of SAA (Fig. 3) was used to assay aliquots of the growth media collected on the different days for the presence of colostrum-SAA.

Cells were kept in culture for 41 days in media supplemented with prolactin. Levels of colostrum-SAA production reached a maximum of almost 3000 ng/ml.

Purification of colostrum associated SAA from cell culture fluid. Colostrum-SAA was purified from cell culture fluid by affinity chromatography. Briefly, an affinity column was prepared by coupling a monoclonal antibody with specificity to SAA to cyanogen bromide activated sepharose 4B. Treating the column with 50 mM Tris, 0.1 NaCl, 0.2 M glycine pH 8 buffer blocked residual active groups on the gel. The column was then washed with 50 mM Tris, 0.1 M NaCl pH 7.2 buffer to remove any excess uncoupled protein. Approximately 50-ml culture fluid was passed over the column. The column was washed with a Tris-NaCl buffer to remove any nonbound proteins that were trapped in the column. The proteins were then eluted from the column with 0.1 M glycine - HCl pH 2.8. The fractions were neutralized immediately. All fractions were assayed by ELISA to determine which fraction contained the maximum amount of colostrum associated SAA and also by western blot to assess the total protein content of the fractions. (Fig. 4).

Determining the amino acid sequence of the purified protein. The fraction containing the greatest amount of the colostrum-SAA was subjected to 12% SDS-PAGE and electroblotted onto a PVDF membrane by a Mini Trans-Blot system (BioRad Laboratories). A section of one lane of the membrane was cut off and was stained with the monoclonal antibody to SAA to verify the presence of the colostrum-SAA. The remainder of the membrane was stained for five minutes in a solution of methanol:water (40:60) containing 0.5% (wt/vol) Bromphenol Blue, and destained in a solution of methanol:water (50:50). The colostrum SAA proteins (identified by the monoclonal antibody stain) were excised from the membrane. The protein was deblocked using pfu pyroglutamate aminopeptidase (TaKaRa Biochemicals) followed by N-terminal sequencing using Edman degradation. Sequencing was performed on a Procise 491 made by PE-Biosystems through the University of Nebraska Medical Center's Protein Core Facility. The N-terminal sequence for colostrum-SAA was present (Figure 5).

Isoelectric Focusing (IEF) of SAA from serum, colostrum and cell culture fluid. The PROTEIN IEF Cell (BioRad) was used for the isoelectric focusing of the various SAA preparations. Ready Strip IPG Strips (BioRad Laboratories) with a pH range of 3-10 were used for the IEF. The second dimension (2-D) of the protein analysis was done by subjecting the IPG strips to 12% SDS-PAGE gel and electroblotting onto a PVDF membrane. The strips and blots were done in duplicate so that one of the blots could be stained with a protein stain, Coomassie Brilliant Blue, (CBB) and the other stained with the anti-SAA monoclonal antibody for the identification of the SAA protein isoforms. The samples also contained internal IEF standards (BioRad Laboratories) so that isoelectric point (pI) of each SAA isoforms could be determined. By comparing the antibody stained spots to the spots of the standards stained with CBB the apparent pI of the SAA isoforms could be determined. All of these procedures were done according to the protocol recommended by the manufacturer.

The proteins subjected to the IEF and 2-D analysis were either affinity purified as described for the cell culture fluid or, in the case of the serum only semi-purified by hydrophobic chromatography. SAA is a highly hydrophobic molecule and will bind readily to Octyl Sepharose beads and then under the appropriate conditions can be eluted off the matrix. Briefly, serum with an elevated SAA level was tumbled for one hour with an equal volume of Octyl Sepharose CL-4B gel to allow for hydrophobic binding of the proteins from the serum to the gel. The gel was washed with Tris-HCl buffer to remove any proteins that were just trapped within the matrix. The proteins bound to the gel were eluted by 60% isopropanol in Tris-NaCl buffer. These eluates contain a variety of proteins of which about 20% is SAA. An aliquot of this preparation or the affinity purified Colostrum-SAA from the cell culture fluid or the colostrum was loaded onto the IPG strips and then standard procedures were followed for the IEF and the 2-D gel.

After analyzing the stained gels it was determined that the colostrum-SAA from both colostrum and MAC-T cell culture fluid had a pI of greater than 8 and was estimated to have an apparent pI of 9.4-9.6. The SAA from the serum contained of three isoforms with apparent pI values of approximately 7.0, 5.8 and 5.5. There was no isoform in the serum that matched the pI of the colostrum-SAA (Fig. 6).

EXAMPLE 6

FUNCTIONAL ROLES OF COLOSTRUM-SAA

A remarkable feature of human physiology is that the mucosal epithelial cells that line the intestinal tract are in contact with a vast number of microbes and yet the incidence of infection and inflammatory complications is low. This suggests that local host protective mechanisms include highly effective, broad-spectrum, non-inflammatory antimicrobial defenses. Whereas the acquired immune system develops an effective response, it does so over a period of days or weeks and in infants the acquired immune system is immature and not fully functional. In contrast, the innate immune system of the intestinal tract is continual or immediately inducible to many potential pathogens introduced into the intestinal tract and brought into close proximity to the mucosal epithelial cells and functional at birth. Intestinal innate immunity includes first-line host-defense elements that range from simple inorganic molecules such as nitric oxide to natural killer cells. There are also a number of molecules produced by the epithelial cells that comprise an effector arm of the innate immune system. These include the relatively small antimicrobial peptides and more complex glycoprotein molecules such as mucins.

Mucins are secreted and cell-surface high-molecular-weight glycoproteins synthesized by epithelial cells of a number of organ systems including the intestinal tract. The strategic interpositioning between the intestinal lumen and the underlying mucosal epithelial cells of the intestinal tract has suggested that mucins have a number of important biological functions. In the intestinal tract, mucins protect against viral infections by inhibition of viral replication and enhancing viral clearance from the intestinal tract. Bacterial pathogens are prevented from adherence to intestinal epithelial cells. Adherence of enteropathogens is the crucial first step required for subsequent invasion, colonization or toxin delivery. Inhibition of adherence of enteric pathogens to intestinal epithelial cells by mucins could be by means of steric hindrance. Applicant's previous work and that of others has also shown that specific mucin-bacterial interactions could also be an important mechanism whereby mucins effect benefit for the host. However, regardless of the mechanism, prevention of mucosal infections is an important function of mucins.

Different mucin genes have been identified and to date, twelve human mucin genes have been identified. However, MUC3 mucin is the predominant intestinal mucin. It has

previously been shown that the MUC3 mucins are effective in preventing the adherence of enteropathogenic *Escherichia coli* (EPEC) to intestinal epithelial cells. Applicant also showed that agents such as non-harmful bacteria that normally colonize the intestinal tract (i.e. probiotics) inhibit EPEC epithelial cell adherence and do so by the upregulation of intestinal mucin genes. It is also well known that breast-feeding infants are far less susceptible to infectious diarrhea than formula fed infants. There are a number of theories why this is so, but it is hypothesized that milk-associated amyloid (colostrum SAA) may be an important inducer of MUC3 gene expression. Applicant has evaluated MUC3 mRNA expression using an in vitro human cell culture assay system. In this system, intestinal cells incubated with the *N*-terminal peptide sequence of colostrum SAA have shown increased MUC3 mRNA expression as compared to control cells grown without the addition of colostrum SAA to the cell culture medium. To further explore this finding, Applicant has evaluated the functional specificity of the *N*-terminal peptide sequence of colostrum SAA by evaluation of MUC3 expression in the same in vitro assay using colostrum SAA *N*-terminal peptide sequences that have been randomly scrambled and a colostrum SAA peptide sequence downstream of the *N*-terminal sequence. If the expression of MUC3 is increased then intestinal cells grown in the presence of colostrum SAA should have a greater capacity to inhibit adherence of bacterial pathogens. This will be studied using EPEC in an in vitro assay system pre-incubated with colostrum SAA in the cell culture medium. Enteropathogenic *E. coli* are non-invasive, non-toxin producing pathogens that have been recognized as a significant cause of diarrhea in third world countries and in day care settings in developed countries. Future studies will evaluate the benefits of colostrum SAA in in vivo studies as well as characterized animal equivalent to human EPEC. Colostrum associated SAA provides a means to naturally upregulate the innate protective mechanisms of the human intestine and would provide a novel form of therapy to a common problem that occurs all too often in the third world leads to death of infants and in the developed world countries and leads to significant morbidity and cost. Furthermore, prevention of intestinal infections for traveler's or those having to live in conditions with altered sanitary practices would also reduce morbidity. Thus, this therapy offers an effective, natural, non-drug/chemical therapy.

In order to address possible functional roles of colostrum-SAA, the Applicant

synthesized, on a standard amino acid synthesizer, a 10 amino acid region of the molecule from bovine that represented the N-terminal portion of the mature protein containing the conserved TFLK. The peptide consisted of the following amino acids: MWGTFLKEAG (SEQ ID NO:30) (Named "N-Terminal"). Since it was anticipated that the TFLK amino acids would be the critical elements of the peptide, we also constructed a peptide in which those four amino acids were scrambled in their order MWGLTKFEAG (SEQ ID NO:28). (Named "Limited Scramble"). For controls we synthesized two peptides, one in which the amino acids in the entire N-Terminal peptide were arranged in random order GKFAWEGMTL (SEQ ID NO:29) (Named "Total Scramble") and a 10 amino acid peptide in which the first 7 were from residues 62-69 of bovine SAA DAAQRGPQQA (SEQ ID NO:30) (Named "C-Terminal").

These four peptides were used in a cell culture assay designed to evaluate them for their properties of inducing mucin (MUC) mRNA production, either MUC3 or MUC2 according to the methods described by Mack et al. (Biochem. Biophys. Res. Commun. 199: 1012-1018, 1994 and Am J Physiol. Vol. 4, part 1, pg. G841-950, 1999).

N-Terminal Peptide Titration MUC3. Intestinal epithelial cells, Mack et al. 1994, 1999, were exposed to the N-terminal 10 amino acid bovine colostrum-SAA peptide (SEQ ID NO:27) at various concentrations for 30 minutes incubation at 37°C. The cells were incubated an additional hour following replacement of the test medium with fresh medium without peptide and then the total cellular mRNA isolated and analyzed for MUC3 specific mRNA. Figure 7 shows that the N-terminal 10 amino acid, bovine Colostrum-SAA "N-terminal" peptide containing the TFLK motif stimulated the production of MUC3 mRNA up to 1-1/2 times that of base line control levels (significance of P-.0002). The optimum concentration was 50 µg/ml medium (see Figure 7).

ANOVA Table for MUC3/28sRNA ration

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
colostrum SAA Concentration	5	87931.344	17586.269	6.670	.0002	33.349	.996
Residual	37	97557.446	2636.688				

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